Entry of influenza A virus: host factors and antiviral targets

Edinger, T O ; Pohl, M O ; Stertz, S

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Entry of influenza A virus – host factors and antiviral targets

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Thomas O. Edinger [#], Marie-Theres O. Pohl [#] and Silke Stertz *

Institute of Medical Virology, University of Zurich, 8057 Zurich, Switzerland

[#] equal author contribution

* corresponding author: Silke Stertz

e-mail: stertz.silke@virology.uzh.ch

phone: +41 44 634 2899

fax: +41 44 634 4967

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SUMMARY

Influenza virus is a major human pathogen that causes annual epidemics and occasional pandemics. Moreover, the virus causes outbreaks in poultry and other animals, such as pigs, requiring costly and laborious countermeasures. Therefore, influenza virus has a substantial impact on health and the global economy. Here, we review entry of this important pathogen into target cells, an essential process by which viral genomes are delivered from extracellular virions to sites of transcription/replication in the cell nucleus. We summarize current knowledge on the interaction of influenza viruses with their receptor, sialic acid, and highlight the ongoing search for additional receptors. We describe receptor-mediated endocytosis and the recently discovered macropinocytosis as alternative virus uptake pathways, and illustrate the subsequent endosomal trafficking of the virus with advanced live microscopy techniques. Release of virus from the endosome and import of the viral ribonucleoproteins into the host cell nucleus are also outlined. Although a focus has been on viral protein function during entry, recent studies have revealed exciting information on cellular factors required for influenza virus entry. We highlight these, and discuss both established entry inhibitors targeting viral and host factors, as well as the latest prospects for designing novel 'anti-entry' compounds. New entry inhibitors are of particular importance for current efforts to develop the next generation of anti-influenza drugs – entry is the first essential step of virus replication and is an ideal target to block infection efficiently.

INTRODUCTION

Influenza A virus (IAV), the causative agent of influenza, is a large burden to the economy and public health world-wide. With waterfowl as the primary reservoir the virus is able to infect a wide variety of birds and mammals, including humans. Due to this trait, zoonotic spillovers occur occasionally and can lead to pandemics with severe consequences for the human population. The swine origin H1N1 virus from the 2009 pandemic and the H5N1 and H7N9 avian influenza viruses are recent examples of animal viruses that acquired the potential to infect and cause disease in humans. A detailed understanding of the viral lifecycle is required to assess or predict the impact of circulating as well as newly emerging viruses but also to develop anti-influenza drugs. The entry process of the virus represents a favorable target for drug development since inhibition of this first step of virus infection should result in an efficient block of virus propagation. One possibility is to target viral proteins essential for entry, e.g. the receptor-binding protein hemagglutinin (HA). An alternative approach is to target cellular proteins required for entry. While in the latter case toxicity could represent an obstacle this strategy would offer the advantage that resistance is less likely to occur. In addition, many viruses use similar entry routes and so it is conceivable that broad-spectrum antivirals could be developed.

IAV belongs to the family *Orthomyxoviridae* and possesses a segmented, negative-sense RNA genome. Unlike most RNA viruses, IAV replicates in the nucleus. Therefore, the virus has to overcome several barriers on its way to the site of replication and, simultaneously, avoid being recognized by the innate immune system. IAV entry is a dynamic process which requires the completion of six individual steps: Attachment to target cells (I), internalization into cellular compartments (II), endosomal trafficking to the perinuclear region (III), fusion of viral

and endosomal membranes (IV), uncoating (V), and import of the viral genome into the nucleus (VI) (fig. 1). Here, we summarize how the virus manages to successfully enter target cells and to transport its genetic material to the nucleus. Furthermore, we discuss which host factors are required by the virus to complete these processes and which inhibitors are available to block individual steps of the IAV entry pathway.

INFLUENZA A VIRUS ATTACHMENT TO HOST CELLS

Sialic acid is the receptor for influenza A virus

The initial step of the viral entry process is the attachment of IAV to the host cell. The primary receptor for IAV is N-acetylneuraminic acid (also called sialic acid) and this receptor is recognized and bound by the viral membrane protein, HA (Palese and Shaw 2007). Sialic acid is the distal residue in oligosaccharide chains of N- and O-linked glycoproteins and –lipids. Often, sialic acid is attached to the underlying galactose by α-2,3 or α-2,6 linkages. This linkage and the resulting structural consequences influence how well IAV can bind to its receptor.

HA is a multifunctional IAV protein mediating virus attachment and fusion. There are 18 different HA subtypes known of which 16 circulate in waterfowl and two subtypes (H17, H18) have been isolated from bats (Tong et al. 2012; Tong et al. 2013). Of note H17 and H18 do not bind to sialic acid; the receptor for these viruses is not yet known (Sun et al. 2013; Tong et al. 2013; Zhu et al. 2013b). HA is expressed as a trimer on the virion surface. The stalk region of HA containing the fusion peptide connects the HA to the virion envelope by a short hydrophobic sequence (Skehel and Wiley 2000). This region is heavily glycosylated on conserved epitopes, which appear to be required for stability and structure of the molecule (Roberts et al. 1993).

71 The globular head is also glycosylated but the glycosylation pattern and -type can be
72 highly variable in different HA subtypes. The receptor binding pocket (RBP) is
73 located on the distal end of the HA trimer at the globular head (fig. 2a) and is highly
74 conserved among different HA subtypes. Mutations in residues of the RBP and those
75 in close proximity can drastically alter the receptor specificity of HA (Connor et al.
76 1994; Liu et al. 2009; Xu et al. 2010). Sialic acid has been shown to occupy the
77 whole RBP and to be the major point of contact between the virus and the cell (Weis
78 et al. 1988). The interaction between sialic acid and HA is believed to be of low
79 affinity. To increase the overall strength of the interaction multiple HA molecules are
80 used to bind to several glycoproteins resulting in high avidity-binding to the cell
81 surface (Sauter et al. 1989).

82 The structure and conformation of HA determines receptor specificity of IAV. It is well
83 established that avian strains prefer sialic acid receptors with a α-2,3 linkage, while
84 human IAV strains generally possess a high receptor specificity for α-2,6 linked sialic
85 acid (Weis et al. 1988; Gamblin et al. 2004; Stevens et al. 2006b). In addition, studies
86 using glycan arrays have shown that modifications on the underlying sugar chains
87 are also recognized by HA and influence the binding of HA to sialic acid (Stevens et
88 al. 2006a). More recent data suggest that the linkage of sialic acid is not the only
89 determinant of receptor binding specificity but that the topological structure of sialic
90 acid contributes to specificity and affinity of HA binding to sialic acid. It was shown
91 that human IAV strains preferentially bind to long, umbrella-shaped sialic acid
92 molecules with α-2,6 linkage, while avian strains generally bind to short sialic acid
93 molecules that adopt a cone-like structure (Chandrasekaran et al. 2008). There are
94 also reports that alternative glycosylations can be recognized by certain IAV strains,
95 e.g. it was shown that N-glycolylneuraminic acid linked to galactose by α-2,3 linkage

96 can serve as receptor for IAV in the duck intestine (Ito et al. 2000).Recent structural
97 studies on receptor binding of H5 and H7 viruses further developed our
98 understanding of differential receptor specificity: For an H5N1 virus that had been
99 selected to transmit in the ferret model it was shown that binding of α-2,6 sialic acid
100 occurred in a similar orientation as in pandemic human viruses (Xiong et al. 2013a).
101 In contrast, the orientation of sialic acid was different when the avian H5 was
102 analyzed in combination with α-2,6 sialic acid. HA from an H7N9 virus that has
103 recently emerged in China was able to bind α-2,6 sialic acid efficiently but in a
104 different orientation compared to human pandemic viruses (Steinhauer 2013; Xiong
105 et al. 2013b). Moreover, these recent studies also suggest that not only efficient
106 binding to α-2,6 sialic acid might be required for human receptor specificity but also a
107 reduction in binding efficiency to α-2,3 sialic acid. For the impact of receptor
108 specificity on tropism, host range and pathogenicity of IAV we refer to (Matrosovich
109 et al. 2009; Imai and Kawaoka 2012; Wilks et al. 2012).

110 Often, virus entry is a multi-step process in which abundant, low affinity receptors are
111 utilized for initial contact of viral particles with the cell. Subsequently, binding of high
112 affinity receptors results in complete attachment and may trigger uptake of a particle.
113 While it is generally accepted that sialic acid is the main receptor for IAV, there is still
114 debate whether IAV requires additional host factors for successful attachment and
115 entry into target cells. It has been observed that IAV binding to sialylated receptors
116 does not always result in internalization of the virus into the host cell (Carroll and
117 Paulson 1985). Furthermore, some desialylated cells retain the ability to bind IAV
118 (Stray et al. 2000; Thompson et al. 2006) indicating that sialic acid might not be the
119 sole receptor required for IAV attachment. Annexin V and 6-sulfo sialyl Lewis X
120 receptors have been proposed as potential additional receptors for IAV attachment

(table 1) (Huang et al. 1996; Gambaryan et al. 2008). Furthermore, IAV was able to attach to but not infect cells with a defect in complex N-glycosylation, suggesting the requirement of an additional factor other than sialic acid for efficient virus infection (Chu and Whittaker 2004). These results were later refined as it was found that proteins containing N-linked glycans are of importance for virus entry via macropinocytosis while clathrin-mediated endocytosis (both discussed later) was not affected by the absence of N-linked glycosylation and that entry of IAV was completely dependent on the presence of sialic acid (de Vries et al. 2012). For dendritic cells and macrophages, there are studies indicating that C-type lectin receptors such as macrophage mannose receptor (MMR), macrophage galactose-type lectin (MGL) and **D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin (DC SIGN) may also act as receptors for IAV entry (Reading et al. 2000; Wang et al. 2008; Upham et al. 2010; Londrigan et al. 2011). However, it remains to be determined whether these receptors alone are sufficient or whether additional co-receptors are required for viral uptake.

Inhibitors of virus attachment

Inhibition of virus attachment might be an attractive strategy for inhibiting IAV infection at the earliest step. Several approaches to block the interaction between HA and sialic acid have been proposed (table 2). Inhibitors can work by either binding to the HA globular head to prevent interactions with the receptor or they can act on the receptor sialic acid. Monoclonal antibodies (mAbs) that bind HA are the most prominent example of inhibitors acting on the virus. Numerous mAbs have been generated and found to inhibit virus replication in cell culture and animal models (reviewed in (Martinez et al. 2009)). Importantly, most mAbs which target the globular head of HA bind and neutralize only the HA they were generated against and a few

146 closely related HAs. However, in recent years broadly neutralizing mAbs that bind
147 conserved epitopes in HA have been developed and these antibodies show promise
148 as inhibitors of many different influenza virus strains (Corti and Lanzavecchia 2013).
149 Two types of antibodies can be distinguished: Antibodies binding conserved epitopes
150 in the globular head (Yoshida et al. 2009; Whittle et al. 2011; Ekiert et al. 2012) and
151 antibodies recognizing conserved sites in the stem of HA (reviewed in (Ekiert and
152 Wilson 2012)). The latter type of antibodies do not inhibit attachment but block fusion
153 and are therefore discussed in the section on viral fusion. Antibodies that recognize
154 conserved epitopes in the globular head can block attachment of different influenza
155 virus subtypes and hold promise for the development of antiviral drugs.

156 Besides the mAbs soluble sialic acid analogues that block the RBP of HA have been
157 suggested as potent inhibitors of IAV infection. Both, soluble α-2,3 and α-2,6 linked
158 sialic acid, can be found in mucus as well as in exosomes released from airway
159 epithelial cells (Baum and Paulson 1990; Kesimer et al. 2009; Roberts et al. 2011).
160 Recent studies propose several synthetic receptor mimics that bind to the RBP for
161 use as antiviral compounds (Kimura et al. 2000; Terabayashi et al. 2006; Nicol et al.
162 2012). It was also shown that sialic acid peptide mimics that bind the RBP of HA can
163 block infection with seasonal H1N1 and H3N2 viruses (Matsubara et al. 2010).
164 Moreover, potent antiviral effects of liposomes loaded with such sialic acid analogues
165 were observed (Hendricks et al. 2013). In addition, the development of receptor-
166 binding compounds that decrease the amount of available binding partners for HA,
167 has also been followed (Matsubara et al. 2009). An interesting strategy to inhibit virus
168 attachment is the use of sialidases, which remove sialic acid from epithelial cell
169 surfaces. DAS181 is a compound consisting of a bacterial sialidase derived from
170 *Actinomyces viscosus* linked to amphiregulin that is currently in phase II clinical trials.

The conjugation of the sialidase to amphiregulin containing an epidermal-growth-factor-like domain is required for the effective targeting of epithelial cells. DAS181 possesses antiviral activity against a broad variety of influenza A and B viruses in cell culture (Nicholls et al. 2013).

ENTRY ROUTES USED FOR INFLUENZA A VIRUS INTERNALIZATION

Internalization of IAV

Upon attachment to the host cell IAV is taken up into the cell. Imaging studies revealed early that the virus enters the cell by receptor-mediated endocytosis (Patterson et al. 1979; Matlin et al. 1981; Yoshimura et al. 1982). It was shown that cold-bound virus was –upon raising the temperature to 37°C- not fusing at the plasma membrane. Virus uptake occurred within minutes after the temperature raise as the half life time of attached viral particles to become resistant to treatment with sialidases was between 10-15 minutes (Matlin et al. 1981). IAV was internalized into mainly clathrin-coated but also into uncoated vesicles. This already suggested that IAV is able to utilize multiple endocytosis routes, not only clathrin-mediated endocytosis. Later, it was demonstrated that the virus can still infect cells defective in clathrin- and caveolin-dependent pathways (Sieczkarski and Whittaker 2002). In line with these data, imaging studies with single viral particles showed that IAV can utilize clathrin- and non-clathrin entry routes in parallel (Rust et al. 2004). Recent studies identified macropinocytosis as alternative pathway exploited by IAV (de Vries et al. 2011). Macropinocytosis refers to the uptake of large-sized cargo through the actin-dependent formation of large endocytic vesicles called macropinosomes. IAV enters the cell by clathrin-mediated endocytosis in the absence of serum and this pathway can be efficiently blocked using the dynamin inhibitor dynasore. However, if serum is

196 present during infection, IAV is taken up into cells by dynamin-dependent and –
197 independent entry routes. A complete block of internalization was only achieved
198 when cells were treated with dynasore in combination with EIPA (de Vries et al.
199 2011). EIPA is an inhibitor of Na^+/H^+ exchangers, which was shown to block
200 macropinocytosis by preventing elevation of the cytosolic pH which in turn affects
201 activation of GTPases required for actin remodeling (Koivusalo et al. 2010). Further
202 studies showed that the choice of entry route is likely to be cell type dependent (De
203 Conto et al. 2011) and that filamentous IAV preferentially uses macropinocytosis for
204 internalization (Rossman et al. 2012). The latter can explain earlier observations on
205 the entry process of filamentous IAV: It had been demonstrated that acidification of
206 endosomes was required but dynamin seemed to be dispensable (Sieczkarski and
207 Whittaker 2005).

208 To date it is not clear whether binding of HA to sialylated glycans is sufficient to
209 initiate internalization of viral particles. Several studies indicate that additional
210 receptors may be required to orchestrate virus uptake (table 1). It was demonstrated
211 that the formation of clathrin-coated pits occurs at faster pace at virus-attached spots
212 than in other areas at the cell surface (Rust et al. 2004). These data indicate that IAV
213 specifically triggers its uptake via clathrin-mediated endocytosis and is therefore
214 likely to interact with additional cell surface receptors to activate downstream
215 signaling processes required for internalization. The adaptor protein Epsin-1
216 localizes to attachment sites of IAV and this coincides with the formation of clathrin-
217 coated pits at that site. In addition, knockdown of Epsin-1 inhibited clathrin-mediated
218 endocytosis of IAV but not of other ligands such as transferrin (Chen and Zhuang
219 2008). Therefore, Epsin-1 is an adaptor recruited specifically for clathrin-mediated

IAV entry indicating that IAV triggers certain pathways that differ from classical clathrin-mediated endocytosis events.

There is growing evidence that receptor tyrosine kinases (RTKs) may play an important role in the uptake of IAV particles. It could be shown that IAV attachment activates EGFR and that activated EGFR promotes virus uptake into target cells. Similar results were obtained for the c-Met kinase so the authors speculate that IAV attachment to the cell surface results in lipid raft formation which serves as signaling platform to trigger RTK activation leading to virus internalization (Eierhoff et al. 2010). Recently, it has been demonstrated that activation of phosphoinositide-specific phospholipase γ1 (PLCγ1) - which acts downstream of EGFR - is required for entry of H1N1 viruses (Zhu et al. 2013a). Interestingly, in this study both, H1N1 and H3N2 viruses were able to activate EGFR following attachment but only H1N1 viruses also activated PLCγ1. These results indicate that different IAV subtypes are capable of specifically activating distinct signaling pathways to facilitate entry. Supporting evidence for involvement of RTKs in IAV entry came from inhibitor studies showing that many RTK inhibitors decrease IAV internalization by macropinocytosis (de Vries et al. 2011). Indeed, N-linked glycans, present on membrane receptors, have shown to be important for IAV entry in the presence of serum (de Vries et al. 2012). Nevertheless, the authors clearly demonstrate that IAV entry was dependent on sialic acid under all experimental conditions. In summary there is evidence for activation of common RTK cascades involving PKC, MEK/ERK and PI3K/AKT by IAV infection, but it remains to be determined how these pathways contribute to virus entry into host cells.

Inhibitors of internalization

Compounds that inhibit virus endocytosis would be of great clinical use as a large array of viruses enter cells by endocytosis. However, most inhibitors used in tissue culture experiments such as dynasore or EIPA are cytotoxic in higher concentrations and prolonged exposure and are therefore not suitable for clinical use. Also RTK inhibitors are problematic as most currently available compounds lack specificity and target a variety of RTK. An interesting compound is Lj001 which affects membrane fluidity and -curvature through (1)02-mediated lipid oxidation. Therefore, its antiviral activity is restricted to enveloped viruses (Wolf et al. 2010; Vigant et al. 2013).

ENDOSOMAL TRAFFICKING OF INFLUENZA A VIRUS

From early to late endosomes

The endosomal system is well described as a cellular sorting system for incoming extracelluar material and intracellular vesicles (reviewed in (Mellman 1996)). Influenza viruses are taken up by endocytosis or macropinocytosis and exploit the transport system via distinct endosomal stages and concomitant changes in pH to release their viral RNPs into the cytoplasm. Upon internalization by either uptake pathway, receptor-mediated endocytosis or macropinocytosis, the virus first localizes to early endosomes and then reaches late endosomes. Endosomal trafficking is known to be a non-linear pathway with a multitude of different branches leading to degradation of extracellular compounds and membrane recycling (Steinman et al. 1983). Here, we focus on how IAV exploits the endosomal pathway.

Before influenza A virus reaches its fusion site it has to pass different stages of the endocytic machinery, which is assembled and constantly renewed around the internalized virus particles (Rust et al. 2004; Cocucci et al. 2012). A difficulty in

detecting these stages of viral trafficking is the short time span viruses remain in the endosomal compartment. Penetration of viral ribonucleoprotein complexes (vRNPs) into the cytoplasm can be detected already after several minutes following virus binding to the cell and vRNPs reach the nucleus within the first hour (Martin and Helenius 1991). For the aim of visualizing viruses along the endosomal trafficking pathway, synchronized infection was established as an important tool that allows monitoring early infection events (Matlin et al. 1981). It has been demonstrated that endosomal trafficking of the virus involves actin- and microtubule-dependent processes (Nielsen et al. 1999; Sun and Whittaker 2007; De Conto et al. 2012). Using single virus trajectories from imaging fluorescently-labelled virions, viral transport was dissected into three different stages (Lakadamyali et al. 2003): First, the virus is transported in the cell-periphery and this process was demonstrated to be actin-dependent. This is followed by the second stage which is marked by rapid dynein-directed movement. Finally, moving of virions along microtubules to the perinuclear region can be defined as stage three. This transport pattern correlates to a large extend with well-established endosomal routes: Early endosomes (EE) containing cargo are transported away from the cell surface via actin dependent transport. EEs are then transported via the motorproteins kinesin-1 and dynein along microtubules towards the nucleus. Simultaneously, EEs constantly exchange vesicles with the trans Golgi network thereby undergoing a maturation process (reviewed in (Huotari and Helenius 2011)). Rab5 and additional proteins such as EEA1 (early endosomal autoantigen 1) and PI(3)K (phosphatidyl-inositol-3-OH kinase), are major regulators of this maturation process and are used as marker proteins to stain EEs (fig. 3) (Bucci et al. 1992; Mu et al. 1995; Simonsen et al. 1998; Christoforidis et al. 1999a; Christoforidis et al. 1999b; Fujioka et al. 2011). Late endosomes (LE) are formed from EEs during their microtubule-dependent transport

into the perinuclear region by acquiring intraluminal vesicles during vesicle exchange with lysosomes or other late endosomes (Luzio et al. 2007; Huotari and Helenius 2011). LE contain integral membrane proteins such as LAMP1 (lysosomal membrane protein 1) and their pH drops down from 6.8-5.9 in EEs to 6.0-4.8 in LE (Maxfield and Yamashiro 1987). The progression from EE to LE is indicated by the so called “Rab Switch” from Rab5 for EE to Rab 7 for LE (Rink et al. 2005). Rab proteins are cellular GTPases that are recruited to vesicle membranes and play a key role in regulating endosomal trafficking. Different Rab proteins are required for different steps in vesicular transport, although some of them are following their endosomal compartments throughout maturation of endosomes (Zerial and McBride 2001).

With respect to IAV infection, both Rab5 and Rab7 have been shown to be required (Sieczkarski and Whittaker 2003). Moreover, protein kinase C βII (PKCβII) has been linked to IAV trafficking in endosomes. Infection of cells pretreated with inhibitors against PKCβII leads to the accumulation of viral particles in LE, without fusion taking place (Sieczkarski et al. 2003). Other important players in LE trafficking are histone deacetylases. Depletion of histone deacetylase 8 (HDAC8) resulted in dysregulation of microtubule organization, centrosome function and maturation of LE to lysosomes and subsequently in a decrease in viral replication (Yamauchi et al. 2011). Furthermore, Cullin 3 which is a scaffolding protein for an E3 ubiquitin ligase complex was shown to be required for IAV entry at the level of LE (Huotari et al. 2012). Importantly, depletion of cullin 3 also inhibited trafficking of other cargos such as epidermal growth factor (EGF) indicating that this pathway is required for transport of a variety of cargos and is not solely used by IAV.

FUSION OF INFLUENZA A VIRUS

Fusion between viral and endosomal membrane

Preceeding the nuclear transport of IAV fusion of viral and endosomal membranes is required to release vRNPs into the cytoplasm. This process is driven by a low pH environment and the class I fusion protein of IAV, the HA. LE posses an acidic environment and thus facilitate the induction of influenza virus HA-dependent fusion at pH 5.0 (Maeda and Ohnishi 1980; Daniels et al. 1985; White and Wilson 1987). Interestingly, HAs of different subtypes display varying pH optima for fusion and HAs of human isolates require lower pHs than avian isolates of the same subtype (Galloway et al. 2013). During the acidification process of endosomes, proton pumps which deliver protons into the endosomal lumen and thereby ensure stepwise acidification, exhibit a crucial function (Galloway et al. 1983; Perez and Carrasco 1994). These so called v-ATPases consist of two complexes, one membrane associated V0 complex and a soluble cytosolic V1 complex, which hydrolyzes ATP as the driving force of acidification (reviewed in (Forgac 2007)). Once IAV is in the acidic environment of LE, HA undergoes conformational changes which expose the fusion peptide and position it towards the endosomal membrane (fig. 2b) (Carr and Kim 1993; Bullough et al. 1994; Chen et al. 1999). It was shown that intermediate stages dependent on pH and membrane proximity exist (Korte et al. 1999; Leikina et al. 2002). Following the final conformational changes, the fusion peptide is inserted into the target membrane which brings the viral and endosomal membranes into close proximity (Tsurudome et al. 1992; Weber et al. 1994; Durrer et al. 1996). Of note, while the crystal structures of pre- and post-fusion HA have been solved the structures of the intermediate stages are not known so far and can only be modeled based on the pre- and post-fusion structures. For the fusion process it was shown

that several HA trimers promote membrane fusion by simultaneous conformational changes and release of folding energy (Markovic et al. 2001). To proceed with fusion between viral and endosomal membranes, HA trimers tilt at the fusion site and the outer leaflets of the membranes interact with each other in a hemifusion stage (Tatulian et al. 1995; Chernomordik et al. 1998). Finally, both membranes fuse and a so-called fusion pore is established (Spruce et al. 1989; Melikyan et al. 1993a; Melikyan et al. 1993b). Through this fusion pore vRNPs can be released into the cytoplasm.

Limited information is available regarding cellular factors required for fusion of IAV. As discussed above, the vATPase complex is essential for acidification of the endosome, a prerequisite for fusion. Only very recently, the tetraspanin CD81 has been identified as another cellular player in the fusion process (He et al. 2013). Approximately 50% of internalized IAV localized to CD81- and Rab5-positive endosomes and fusion was observed to occur in these vesicles. In the absence of CD81 fusion was reduced by 50%. It is currently unknown how CD81 contributes to fusion but CD81 seems to mark a productive entry route for IAV.

Inhibitors of fusion

Inhibition of viral fusion can be achieved by inhibition of acidification in endosomes (table 2). One of the most potent inhibitors for this purpose is Bafilomycin A1 (BafA1). Bafilomycins belong to the family of macrolide antibiotics that were shown to inhibit vacuolar-type proton pumps involved in viral entry (Bowman et al. 1988; Ochiai et al. 1995). Similar effects were shown for diphyllin and SaliPhe, other v-type ATPase inhibitors (Huss and Wieczorek 2009; Konig et al. 2010; Muller et al. 2011). Small molecules that bind to the stem region of HA and thereby hinder the conformational changes required for fusion represent another class of fusion inhibitors. The first

compound of this group to be discovered was TBHQ which has been crystallized in complex with HA (Bodian et al. 1993; Russell et al. 2008). Later on, several compounds that act in a similar way have been described: BMY-27709, CL-385319, RO5464466, stachyflin and 4c (Luo et al. 1997; Plotch et al. 1999; Yoshimoto et al. 1999; Vanderlinden et al. 2010; Zhu et al. 2011). Unfortunately, resistance mutations in HA can develop rapidly within few passages of the virus and confer resistance to this type of compound. Arbidol has also been identified as an inhibitor of membrane fusion. Mutations rendering viruses resistant to arbidol have been mapped to HA and seem to impact acid stability of HA (Leneva et al. 2009). Of note, arbidol is approved as anti-influenza drug in Russia. Alternatively, the stem region of HA can be targeted by broadly neutralizing antibodies as mentioned above (Corti and Lanzavecchia 2013). Such antibodies bind to a region of HA that is conserved even between different subtypes and this enables the antibodies' potential to inhibit many different strains of IAV (Okuno et al. 1993; Throsby et al. 2008; Ekiert et al. 2009; Sui et al. 2009; Corti et al. 2011). While they do not prevent binding to the host cell they interfere with the conformational changes required for fusion. These antibodies represent promising drug candidates but also could help to design a vaccine that provides protection against a broad range of IAV strains.

UNCOATING

Release of vRNPs into the cytoplasm

Upon fusion of viral and endosomal membranes IAV uncoating is completed with the release of the viral RNPs into the cytosol. This process requires coordinated action of the viral proteins M2 and M1. In the intact virus particle M1 forms a structured layer underneath the viral membrane which can be visualized by electron microscopy

(Ruigrok et al. 2000; Calder et al. 2010; Fontana et al. 2012; Fontana and Steven 2013). It is assumed that M1 plays a crucial role for the architecture of the virion by linking the viral membrane containing the glycoproteins with the RNPs in the virus core. While the expression of viral glycoproteins can result in production of virus-like particles even in the absence of M1 the matrix protein is required for production of infectious virions (virus assembly is reviewed in (Rossman and Lamb 2011)). This is supported by the observation that M1 determines the shape of the virion: Exchange of M1 is sufficient to change the morphology of virions from spherical to filamentous (Roberts et al. 1998; Bourmakina and Garcia-Sastre 2003; Elleman and Barclay 2004). It is currently unclear how M1 interacts with the viral membrane and/or glycoproteins; specific binding domains have not yet been mapped (Zhang and Lamb 1996; Schmitt and Lamb 2005). For the interaction of M1 with the vRNPs it could be demonstrated that the middle domain of M1 is responsible for binding to NP on the RNP (Noton et al. 2007).

During the uncoating process the interaction of M1 with the viral membrane as well as the interaction of M1 with the vRNPs has to be released in order to allow complete uncoating and subsequent transport of the RNPs into the nucleus. This requires the activity of the viral protein M2. M2 was identified in 1981 as the second protein encoded by segment 7 of IAV (Lamb and Choppin 1981). It was found to form tetramers that are present in virions (Zebedee and Lamb 1988; Holsinger and Lamb 1991; Sugrue and Hay 1991) and it was described to possess ion channel activity selective for monovalent ions (Pinto et al. 1992; Chizhmakov et al. 1996). Interestingly, the ion channel activity of M2 is regulated by pH: With lower pH the ion channel activity increases and histidine 37 of M2 is crucial for this regulation (Pinto et al. 1992). The transmembrane pore of the channel is lined by a series of amino

419 acids that all lie on the same side of an alpha-helix; four of these helices from the
420 four monomers form the channel (Grambas et al. 1992; Pinto et al. 1992; Wang et al.
421 1993; Stouffer et al. 2008). During the entry process of IAV the ion channel activity of
422 M2 is required for uncoating: Upon acidification of the endosome M2 mediates proton
423 influx from the endosome into the virion resulting in a decrease of the pH within the
424 virus particle (Wharton et al. 1994). This M2-mediated change in pH is required for
425 the detachment of M1 from the vRNPs resulting in the release of the vRNPs into the
426 cytoplasm (Zhirnov 1990). Furthermore, it was observed that M1 separates from the
427 RNPs before they are imported into the nucleus (Bukrinskaya et al. 1982; Martin and
428 Helenius 1991). Interestingly, this initial RNP nuclear import can be blocked by
429 expression of M1 but brief low pH treatment can in turn relieve the block mediated by
430 M1 (Bui et al. 1996). These observations have lead to the current model in which the
431 pH drop in the virion within the endosome causes conformational changes in M1 and
432 subsequently the interaction between the RNPs and M1 is weakened or lost. The
433 changes in M1 conformation have been visualized by electron microscopy: The
434 helical structure of the M1 layer in the virion is lost in acid-treated virions (Calder et
435 al. 2010; Fontana et al. 2012). Before the loss of the M1 structure rearrangements in
436 the M1 layer could be detected (Fontana and Steven 2013). It is currently unclear
437 how the conformational change occurs but it was suggested that the linker region
438 between the N- and C-terminal domain of M1 is important: *In vitro* the linker peptide
439 changed its conformation upon pH drop but only in the presence of zinc ions, which
440 have been detected in influenza virions (Elster et al. 1994; Okada et al. 2003). Not
441 much is known about the involvement of cellular factors in this process yet. Very
442 recently, the E3 ubiquitin ligase Itch was reported to be required for efficient
443 uncoating (Su et al. 2013). The authors could demonstrate that Itch gets

444 phosphorylated and recruited to endosomes upon IAV infection where it ubiquitinates
445 M1 and thereby facilitates release of the vRNPs.

446 **Inhibitors of uncoating**

447 Amantadine is the best known example for an inhibitor of M2. Its antiviral activity was
448 first described in 1964 (Davies et al. 1964). Later it was found that it targets the M2
449 protein of influenza A virus and thereby exerts its antiviral function (Skehel et al.
450 1978; Hay et al. 1985). Rimantadine is structurally similar and also blocks M2;
451 together this drug class is called adamantanes. Unfortunately, resistance to the
452 adamantanes can be achieved by just a single amino acid change in M2 and this has
453 no or very little impact on viral fitness (Hay et al. 1986; Hayden et al. 1991; Sweet et
454 al. 1991). Moreover, resistance to the adamantanes is widespread since the
455 beginning of the 21st century and therefore current guidelines do not recommend the
456 use of adamantanes (Bright et al. 2005). A recent study reported on the development
457 of novel M2 inhibitors that can block adamantane-sensitive as well as resistant
458 strains (Wang et al. 2013). It is therefore conceivable that novel M2-inhibiting drugs
459 might become available for clinical use in the future (table 2).

460

461 **NUCLEAR IMPORT OF VIRAL RNPs**

462 **Import of vRNPs into the nucleus**

463 After completion of the uncoating process the RNPs are transported into the nucleus.
464 Already early studies observed that NP accumulates in the nucleus while M1
465 distributes between cytoplasm and nucleus (Martin and Helenius 1991). Given the
466 size of the vRNPs it was hypothesized that an active, energy-dependent process
467 would mediate their import. Of note, also RNPs microinjected into the cytoplasm of

cells were capable of entering the nucleus (Kemler et al. 1994). In 1995, O'Neill and co-workers demonstrated that the viral RNA was not able to enter the nucleus; addition of NP was required. Moreover, they could show that at 0°C NP docks to the nuclear envelope in the presence of karyopherins and is imported into the nucleus upon addition of the cellular import factors Ran and p10 proteins and a temperature shift to 20°C (O'Neill et al. 1995). This clearly showed that viral RNPs are imported via the cellular karyopherin import pathway. All protein components of the RNP, the three polymerase subunits and NP, possess nuclear localization signals (NLS). Nevertheless, import of the RNP only depends on the NLS in NP (O'Neill et al. 1995; Cros and Palese 2003). First, an unconventional NLS in the N-terminus was described in NP (Wang et al. 1997). Later on, a second bi-partite NLS was identified between amino acids 198-216 (Weber et al. 1998) as well as a third one around amino acids 320-400 (Bullido et al. 2000). For import of the RNPs the unconventional NLS seems to be the most important one (Cros and Palese 2003). On the cellular side karyopherins alpha 1, alpha 3 and alpha 5 have been identified as the main importins for RNPs (table 1) (O'Neill et al. 1995; Wang et al. 1997; Melen et al. 2003). Also CSE1L, a cellular factor required for cycling of karyopherins between nucleus and cytoplasm, has been shown to be required for import of RNPs early in infection (Konig et al. 2010). Later in infection when the individual polymerase proteins get imported the NLS on the polymerase subunits become important and other karyopherins are involved. This topic is reviewed in (Hutchinson and Fodor 2012). Upon import into the nucleus the karyopherins bind to RanGTP, which results in release of cargo and this marks the end of the viral entry process.

OUTLOOK

Entry of IAV into target cells is the very first step of the viral life cycle and as such is crucial for the establishment of infection. The receptor specificity of the virus' HA determines tropism of the virus, thereby contributing to outcome of disease, and potentially virus spread between susceptible hosts. In recent years our understanding of the differential receptor specificity between avian and mammalian influenza viruses has greatly improved and exciting structural insights have been obtained. However, more work is still required to fully understand and predict receptor specificity of all HA subtypes. For entry into target cells the virus relies on and exploits existing cellular pathways of transporting cargo, thus the entry process is a complex interplay between virus and host cell. Advances in live cell microscopy are of great value in tracking virions during entry in real-time and in monitoring the interaction of the virus with cellular factors and compartments. Novel findings indicate that virus uptake and -trafficking may not be equal to that of other cargo transported into the cell. Instead, IAV specifically recruits factors facilitating entry and activates signaling molecules such as RTKs within minutes after infection. Future studies will shed light on how these host factors contribute to virus entry on a molecular level. The increasing insight into these processes can be exploited to develop means of inhibiting the virus early in infection. Novel treatment options may be specifically directed against IAV, or be of broad antiviral efficacy if targeting entry routes used by several viruses. In the near future, we may obtain a detailed insight into the cell biology of IAV entry and profit from newly developed antivirals targeting host factors rather than viral proteins, thereby minimizing the occurrence of resistance as observed with the M2 and NA inhibitors.

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Figure legends

Fig. 1: Schematic representation of the influenza A virus entry process.

Fig. 2: Structure of hemagglutinin

a) Structure of the hemagglutinin of influenza A virus A/SouthCarolina/1918 based on (Gamblin et al. 2004; PDB accession no. 1RUZ). The trimeric complex of HA is shown with one monomer highlighted in colour. HA1 is depicted in red, HA2 in blue and the receptor binding site in green.

b) The pre- and post fusion conformations of HA are shown (Bullough et al. 1994; PDB accession no. 1HTM). For the post fusion conformation only the structure of the part represented in blue could be resolved. HA1 was not included in the structure and was modelled on according to (Palese and Shaw 2007).

Fig. 3: Super resolution microscopy of influenza A virus in endosomes

A549 lung epithelial cells were infected with influenza A virus (A/WSN/33, MOI of 25) for 30-180 minutes. Virus was added to the cells in an inital cold binding step to synchronize the infection process. Cells were fixed and stained for DAPI and NP (a), DAPI, NP and EEA1 (b1) or NP and EEA1 (b2-b4). Images were acquired by standard immunofluorescence microscopy (CLSM – confocal laser scanning microscopy, a, b1) or super resolution microscopy (STED – stimulated emission depletion, b2-b4). In b3-b4 rendered (IMARIS) images of viral particles within endosomes are shown. In b4 the transparency of the endosomal staining was increased to allow visibility of viral particles inside the respective endosome.

Table 1: Host factors involved in IAV entry

host factor	entry strep	reference
sialic acid	attachment	Palese and Shaw, 2007
C-type lectins	attachment	reviewed by Londrigan *et al.*, 2011
annexin V	attachment	Huang *et al.*, 1996
6-sulfo sialyl Lewis X	attachment	Gambaryan *et al.*, 2008
dynamin	internalization	Roy *et al.*, 2000(Roy et al. 2000)
actin	internalization	Gottlieb *et al.*, 1993(Gottlieb et al. 1993)
clathrin	internalization	Matlin *et al.,* 1981
epsin-1	internalization	Chen *et al.*, 2008
EGFR	internalization	Eierhoff *et al..*, 2010
c-Met kinase	internalization	Eierhoff *et al.*, 2010
PLC-γ1	internalization	Zhu *et al.,* 2013
Rab 5	endosomal trafficking	Sieczkarski *et al.*, 2003
Rab7	endosomal trafficking	Sieczkarski *et al.*, 2003
PKC βII	endosomal trafficking	Sieczkarski *et al.*, 2003
cullin 3	endosomal trafficking	Huotari *et al.*, 2012
HDAC8	endosomal trafficking	Yamauchi *et al.*, 2011
vATPase	endosomal acidification	Guinea *et al.*, 1995(Guinea and Carrasco 1995)
CD81	fusion	He *et al.,* 2013
ITCH	uncoating	Su *et al.*, 2013
karyopherin (α1; α3; α5)	import	Wang *et al.*, 1997
Ran	import	O'Neill *et al.*, 1995
p10	import	O'Neill *et al.*, 1995
CSE1L	import	Konig *et al.*, 2010

Table 2: Inhibitors of IAV entry

inhibitor	entry step	potential as drug	reference
mAbs (HA-RBP)	attachment	yes	reviewed by Clementi *et al.*, 2012 (Clementi et al. 2012)
SA mimics	attachment	yes	reviewed by Vanderlinden *et al.*, 2013 (Vanderlinden and Naesens 2013)
SA binders	attachment	yes	reviewed by Vanderlinden *et al.*, 2013
Sialidases e.g. DAS181	attachment	yes/phase II trial	reviewed by Nicholls *et al.,* 2013
Dynasore	internalization	laboratory use	De Vries *et al.*, 2011
EIPA	internalization	laboratory use	De Vries *et al.*, 2011
Receptor tyrosine kinase inhibitors	internalization		Eierhoff *et al.*, 2010 and De Vries *et al.*, 2012
Lj001	internalization	yes	Wolf *et al.*, 2010
Bafilomycin A1	endosomal acidification	laboratory use	Guinea *et al.*, 1995
mAbs (HA stalk)	fusion	yes	reviewed by Corti *et al.*, 2013 (Corti and Lanzavecchia 2013)
Small molecule inhibitors (HA stalk)	fusion	yes	reviewed by Vanderlinden *et al.*, 2013
Amantadine	uncoating	approved	Davies *et al.*, 1964
Rimantadine	uncoating	approved	Rabinovich *et al.*, 1969 (Rabinovich et al. 1969)
benzyl-substituted amantadine derivatives	uncoating	yes	Wang *et al.*, 2013
importazole	import	laboratory use	Chou *et al.*, 2013 (Chou et al. 2013)

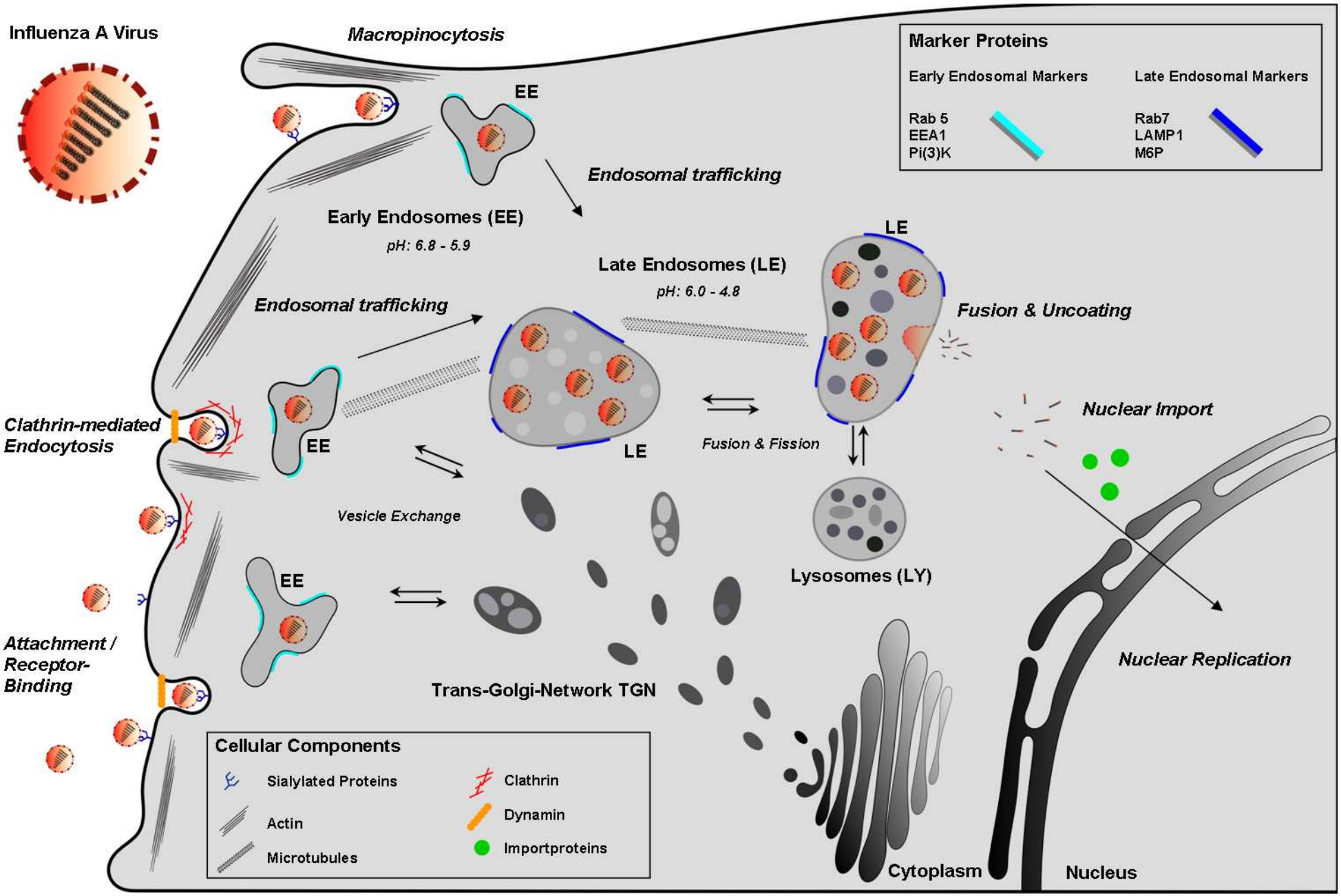
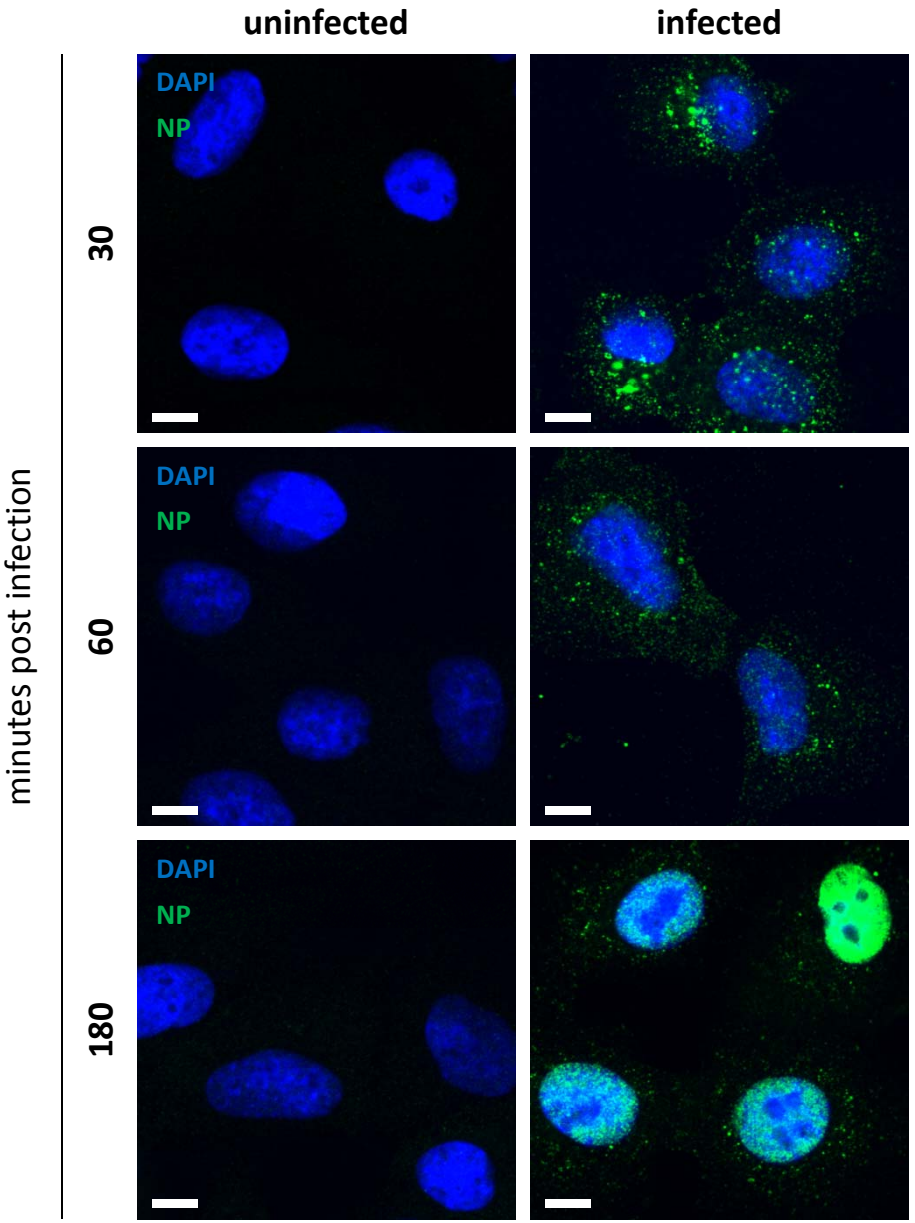
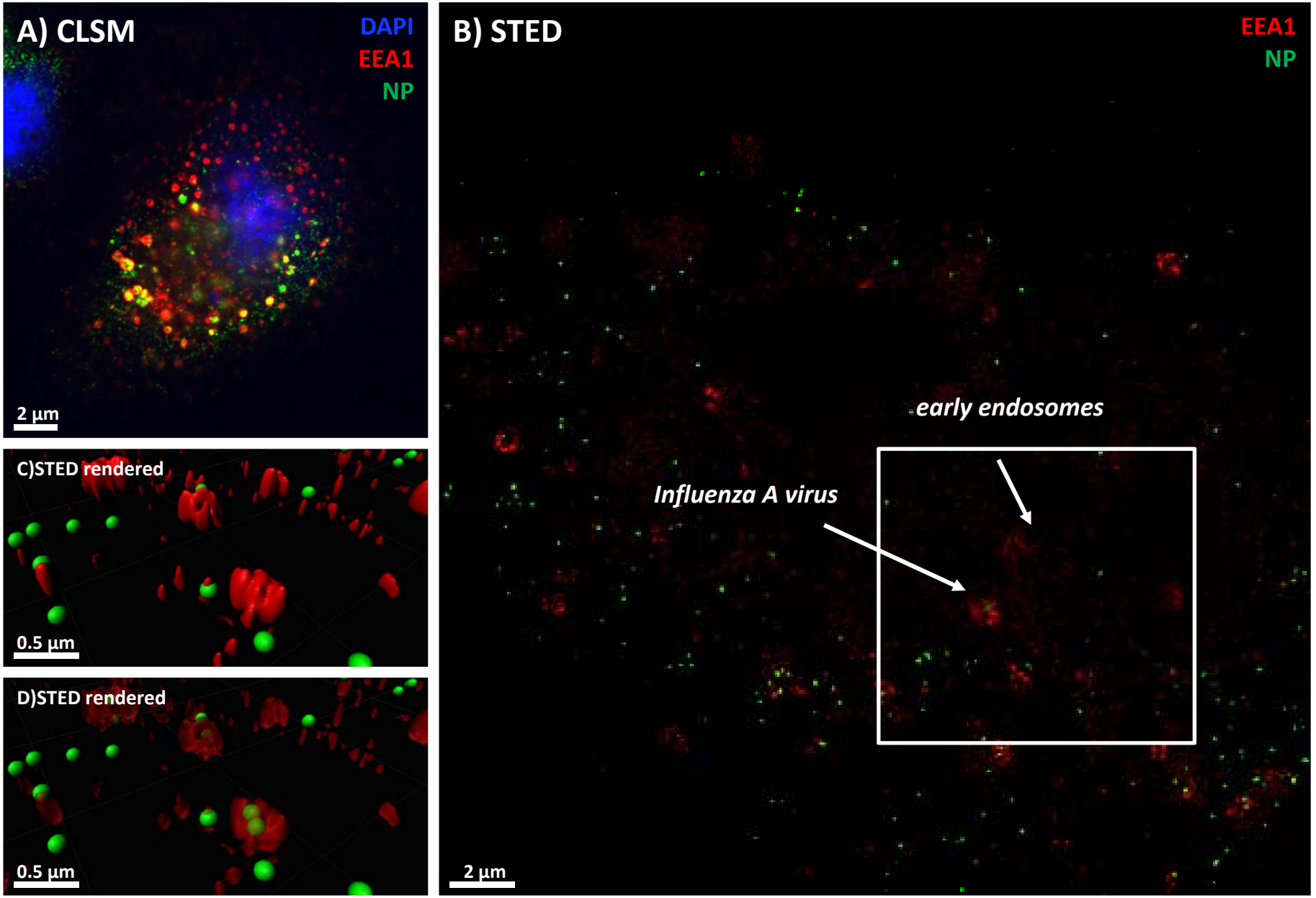

Influenza A Virus
Macropinocytosis
EE
Endosomal trafficking
Early Endosomes (EE)
pH: 6.8 - 5.9
Late Endosomes (LE)
pH: 6.0 - 4.8
LE
Endosomal trafficking
Fusion & Uncoating
Clathrin-mediated Endocytosis
EE
LE
Fusion & Fission
Nuclear Import
Vesicle Exchange
Lysosomes (LY)
EE
Attachment / Receptor-Binding
Trans-Golgi-Network TGN
Nuclear Replication
Cytoplasm
Nucleus
Marker Proteins
Early Endosomal Markers
Rab 5
EEA1
Pi(3)K
Late Endosomal Markers
Rab7
LAMP1
M6P
Cellular Components
Sialylated Proteins
Actin
Microtubules
Clathrin
Dynamin
Importproteins

Figure 2: Kinetics of IAV entry



A549 lung epithelial cells were infected with Influenza A virus (A/WSN/33, MOI of 25) for 30-180 minutes. Virus was added to the cells in an inital cold binding step to synchronize the infection process. Cells were fixed at the indicated times p.i. and immunofluorescence microscopy was carried out for NP and DAPI signal. Scale-bar equals 10 µm.

Figure 3: Super resolution microscopy of influenza A virus in endosomes



A549 lung epithelial cells were infected with influenza A virus (A/WSN/33, MOI of 25) for 60 minutes. Virus was added to the cells in an inital cold binding step to synchronize the infection process. Cells were fixed and (A) standard immunofluorescence microscopy (CLSM – confocal laser scanning microscope) for DAPI, NP and EEA1 signal or (B-D) super resolution microscopy (STED – stimulated emission depletion) for NP and EEA1 was carried out. In C/D rendered (IMARIS) images of viral particles within endosomes are shown. In D the transparency of the endosomal staining was increased to allow visibility of viral particles inside the respective endosome.